RESEARCH REPORT

Evaluation of Physiological Amino Acids Profiling by Tandem Mass Spectrometry

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Abstract Background: Nowadays, the most conventional method to quantify physiological amino acids consists in ion exchange chromatography (IEC) followed by post-column ninhydrin derivatization and UV detection at two wavelengths. Unfortunately, the technique presents some drawbacks such as long run time, large sample volume, and specific costs associated to the maintenance of a dedicated instrument. Therefore, we aimed to switch towards a mass spectrometry approach.

Methods: We have tested the aTRAQ kit for Amino Acid Analysis of Physiological Fluids (AB Sciex), affording the selective quantification of about 40 amino acids, and present here the results of our assessments.

Results: Outlined accuracy profiles for each amino acid demonstrated very reliable data. A good linearity was observed from 1 to 1,000 μmol/L. Results comparison with IEC showed a right concordance. Reference intervals established were very similar to those obtained by IEC and patients suffering from inborn error of metabolism have been readily identified.

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Conclusions: The present approach offers a valid alternative to the IEC method, with several advantages: lower sample volume, run time reduction, and improved specificity. However, the aTRAQ method requires minute data reviewing, expending the overall time of procedure. Finally, financial and practical considerations of both techniques have to be counterbalanced before engaging any transition.

Abbreviations

1MHis	1-Methylhistidine
3MHis	3-Methylhistidine
Aad	Alpha-Aminoadipate
Abu	Alpha-Aminobutyrate
Ala	Alanine
Ans	Anserine
Arg	Arginine
Asa	Argininosuccinate
Asn	Asparagine
Asp	Aspartate
bAib	Beta-Aminoisobutyrate
bAla	Beta-Alanine
BSA	Bovine Serum Albumin
Car	Carnosine
Cit	Citrulline
Cth	Cystathionine
Cys	Cystine
ERNDIM	European Research Network for evaluation and improvement of screening Diagnosis and treatment of Inherited disorders of Metabolism
EtN	Ethanolamine
GABA	Gamma Aminobutyrate
Gln	Glutamine
Glu	Glutamate
Gly	Glycine

Hcit	Homocitrulline
Hcy	Homocystine
His	Histidine
HPLC	High Pressure Liquid Chromatography
Hyl	Hydroxylysine
Hyp	Hydroxyproline
IEC	Ion Exchange Chromatography
IEM	Inborn Error of Metabolism
Ile	Isoleucine
IS	Internal Standard
Leu	Leucine
Lys	Lysine
Met	Methionine
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
Nle	Norleucine
Nva	Norvaline
Orn	Ornithine
PEtN	Phosphoethanolamine
Phe	Phenylalanine
Pro	Proline
PSer	Phosphoserine
Sar	Sarcosine
Ser	Serine
Tau	Taurine
Thr	Threonine
Trp	Tryptophane
Tyr	Tyrosine
Val	Valine

Introduction

Amino acid disorders represent an important field in the wide classification of inborn errors of metabolism (WHO 2010). Such conditions result from a defect in the metabolic pathways of amino acids, leading to the accumulation of the corresponding metabolite in plasma, urine, cerebrospinal, amniotic fluid, or other biological matrix.

Besides the conventional 20 proteinogenic amino acids, several nonstandard amino acids hold critical physiological functions. Some of them are the result of posttranslational protein modification (i.e., hydroxyproline), which is an essential process for the function or regulation of proteins, while others are intermediates of metabolic pathways (i.e., urea cycle intermediates) and they also act as neurotransmitters (i.e., GABA). Acknowledging the central role of amino acids in protein synthesis and metabolism homeostasis, profiling of these markers has became a noticeable tool in the identification and follow-up of primary disorders. The biochemical findings that may be present in amino acid disorders include metabolic acidosis, hyperammonemia, hypoglycemia with appropriate or

increased ketosis, multi-system disorder, developmental delay, encephalopathy, coma, or death.

Amino acids are not only associated with inborn errors of metabolism, they are also sensitive markers of the nutritional state and of the function of various organs such as the liver, the kidneys, the intestine, or the muscles. These dysfunctions generate subtle alterations in amino acid concentrations; therefore, accuracy of the amino acid analysis is a crucial matter in order to interpret these changes.

In those contexts' workup, the most conventional method for amino acid quantification consists in ion exchange chromatography (IEC) followed by post-column ninhydrin derivatization and UV detection at two wavelengths. Actually, amino acid analyzers have been used since more than 50 years in biochemical laboratories and still are considered as a reference method (Dietzen et al. 2008; Duran 2008). Notwithstanding, the technology suffers some drawbacks; chromatographic separation lasts about 150 min and requires high sample volumes (>100 μL). In addition, running analysis on dedicated instruments, requiring expensive maintenance costs, depicts another leading concern.

Considering the disadvantages of IEC technique and owning a triple quadrupole mass spectrometer for the newborn screening of inborn errors of metabolism, we wanted to evaluate a mass spectrometry approach. Nowadays, identification and quantification of amino acids by mass spectrometry are rather uncommon in clinical laboratories. Only a few methods have already been developed and validated and are based either on native (Piraud et al. 2003, 2005; Waterval et al. 2009) or on derivatized (Dettmer et al. 2012; Dietzen et al. 2008; Harder et al. 2011; Held et al. 2011) metabolites identification.

Based on this perception, we have tested the aTRAQ kit for Amino Acid Analysis of Physiological Fluids (AB Sciex), affording the selective quantification of about 40 amino acids. We report here the results of our assessment, based on plasma, urine, and cerebrospinal fluid (CSF) samples.

Material and Methods

Chemicals

aTRAQ kit for Amino Acid Analysis of Physiological Fluids was provided by AB Sciex. Mixes of amino acid standards (reference numbers A6407 and A6282), glutamine and sodium chloride (NaCl) were obtained from Sigma. Bovine serum albumin (BSA) was purchased at Roche.

Samples

Five-point calibration curves were prepared by diluting amino acid standard mixes and glutamine in 75 g/L BSA, isotonized with 0.9 g/L NaCl. Final calibration concentrations were the following: 1,000, 250, 100, 50, and 10 μmol/L for all amino acids, except for cystine whose concentrations were 500, 125, 50, 25, and 5 μmol/L.

Validation samples were prepared in the same way with final concentrations at 1,000, 200, 75, 20, 5, and 1 μmol/L for all amino acids, except for cystine whose concentrations were 500, 100, 37.5, 10, 2.5, and 0.5 μmol/L. Samples from the ERNDIM Amino Acids Scheme have also been used for method validation; target concentrations used corresponded to the consensus mean concentrations of all participating laboratories.

Plasma, urine, and CSF samples were gathered from normal individuals.

Sample Derivatization

Conceptually, amino groups are labeled with tags of varying isotope patterns. The label consists of a reporter group (with the masses m/z 113 or 121), a neutral linker, and an amino-reactive group (N–hydroxysuccinimide ester). The 121-labeling reagent is used to derivatize the amino acids in the sample, whereas the 113-labeling reagent is employed to provide a 113-labeled standard mix containing 42 analytes for absolute quantification.

Briefly, sample (40 μL) proteins were precipitated with 10 % sulfosalicylic acid (10 μL) containing 400 μmol/L norleucine. After vortexing and centrifugation, the supernatant (10 μL) was mixed with 40 μL labeling buffer containing norvaline. At this moment, part of the mixture (20 μL) can be set aside if allo-isoleucine quantification is necessary. To 10 μL of this reaction mix, 5 μL of isopropanol diluted aTRAQ Δ8-reagent was added. After 30-min incubation at room temperature, 5 μL of hydroxylamine was added and homogenized mixture was incubated 15 min at room temperature. Then, 32 μL of reconstituted aTRAQ Internal Standard solution was added to the reaction mixture. If allo-isoleucine testing is mandated, 5 μL of underivatized mix, saved from the second step, is added. The specimen is then partly concentrated under nitrogen for 15 min. Finally, half-evaporated samples were diluted 10 times with milli-Q water before injection.

Allo-isoleucine coelutes with isoleucine when labeled with the aTRAQ Δ8-reagent. Therefore, for confirmation and follow-up of maple urine syrup disease (MSUD) patients, allo-isoleucine is quantified in its underivatized form, which is then chromatographically well separated from isoleucine.

Separation and Detection

Derivatized samples (1 μL) were introduced into a TQ5500 tandem mass spectrometer (AB Sciex) using a Prominence AR HPLC system (Shimadzu).

Tagged amino acids were chromatographically separated on the AB Sciex C18 column at 50 °C. The column dimension was 15 cm long, with a 4.6 mm inner diameter. A binary gradient of water (mobile phase A) and methanol (mobile phase B), both containing 0.1 % formic and 0.01 % heptafluorobutyric acids, was flowing through the system at 0.8 mL/min. The percentage of mobile phase B was gradually increased from 2 % to 90 % in 13 min, followed by a 5-min re-equilibration step.

Acquisition in the mass spectrometer was achieved by multiple reaction monitoring (MRM) using the scheduled MRM algorithm (See Supplemental Data Table 1). This latter functionality eliminates the requirement of multi-period experiments by monitoring each transition only across its expected elution time. MRM transitions are then organized automatically, optimizing dwell time, cycle time, and detection window.

Data recording and treatment were performed with the v.1.6 Analyst software (AB Sciex).

Quantification

As recommended by manufacturer, quantification was achieved dividing the analyte area by the IS area and then multiplying by the IS concentration. To evaluate the performance of such calibration protocol, we compared concentrations estimated by this internal quantification method with results calculated by a five-point external calibration curve.

Analytical Validation

The method was validated using accuracy profiles based on β-expectation tolerance intervals for the total error measurement and assessing the measurements' uncertainty (Gustavo Gonzales and Angeles Herrador 2006; Hubert et al. 2004; Hubert et al. 2007a, b; Rozet et al. 2006). For this purpose, five measurements of each validation samples have been analyzed in five independent series, and before each run, a calibration curve was tested twice. Computing of validation data was ensured using the v3.0 Enoval validation software (http://www.arlenda.com).

Method Comparison

Comparison was done between the MS method and the ion exchange chromatography (IEC) amino acid analyzer. Five independent measurements of 4 ERNDIM Amino Acids

Scheme samples, covering the main part of the physiological range, have been carried out. The ERNDIM "IEC with Ninhydrin and one Internal Standard" method has been considered for this purpose, and the IEC target result used for method comparison is therefore the consensus mean of more than 100 laboratories.

Slope and intercept were estimated by Passing and Bablok regression with Medcalc v12.1 software.

Reference Ranges

Normal population values have been set up for plasma, urine, and CSF on several age categories: 0–2 years, 2–12 years, and >12 years. Intervals, proceeding from about 20 to 30 samples in each age class, have been generated using nonparametric analysis and represent the central 90 % (5–95 %) of the populations evaluated.

Clinical Evaluation

Samples (urine and plasma) from patients with confirmed inborn error of metabolism have been tested. The assessment included urines from homozygote cystinuria, Non ketotic hyperglycinemia, tyrosinemia I, and cystathioninuria. Plasmas from treated phenylketonuria, maple syrup urine disease, and ornithinemia patients have also been tested.

Results

Internal Versus External Calibration

To compare the internal quantification versus the external five-point calibration curve, each back-calculated concentration was normalized, in percentage, against its expected value. These recovery results were then assayed with a one-sample t-test to evaluate whether the average of observations differs significantly from 100 %.

External calibration gave slightly better recovery results for most amino acids. This difference turns out to be very significant for the upper concentration (1,000 μmol/L). Therefore, all further results have been back calculated against this external calibration curve, which was reanalyzed twice before each run. Additionally, opting for this quantification way helps to decrease the batch-to-batch variability (Held et al. 2011).

Method Capacities

An approach using accuracy profiles based on tolerance intervals for the total error measurement, including both bias and standard deviation for intermediate precision, was applied to demonstrate the method capability. Method is considered as valid within the range for which the accuracy profile is fully included inside the accuracy acceptance limits, set at ±30 % for upper concentrations (>75 μmol/L) and at ±50 % for lower concentrations (≤75 μmol/L). The β-expectation tolerance interval, which describes a region where, on average, a proportion β of future measurements will fall, was fixed at 5 %. This proceeding gives the guarantee that each further measurement of unknown samples will be included within the tolerance limits at the fixed level and thus within the acceptance limits.

Accuracy profiles have been generated for all amino acids, and a profile example is provided in Fig. 1. Based on these profiles, the lower limit of quantitation (LLOQ) is defined as the smallest quantity of each amino acid that fits within acceptance range (Fig. 2 and Supplemental Data Table 2).

Accuracy profile can be used as a visual decision tool to assess the validity of an analytical method. However, as for every graphical representation, this visual interpretation is partly subjective. Therefore, different "desirability" indexes have been considered: dosing range index, precision index, and trueness index (Rozet et al. 2007). The dosing range index is defined as the ratio between the length of interval in which the method is considered as valid and the length of the interval between the highest and lowest concentration levels investigated during the validation. The precision index corresponds to the ratio between the area defined within the acceptance limits and the limits of β-expectation tolerance interval, and the area bounded by the upper and lower acceptance limits, for which the method is considered as valid. The trueness index is defined as 1 minus the ratio between the sum of the square of the observed bias at each concentration level and the sum of the square of the maximum tolerated bias at each concentration level, for which the method is considered as valid. All these three indexes vary from 0 to 1; the better is the method for a criteria, the closer to one is the corresponding index (See Supplemental Data Statistic) (Rozet et al. 2007).

A synthetic radar chart of each index has been built for each amino acid (Fig. 3). Dosing range of bAla, Pser, and Sar are narrower than for other amino acids. For Sar, this can be explained by the saturation of the instrument detector at higher concentrations (1,000 μmol/L). Nevertheless, clinical impact of such phenomenon is reduced; physiological concentrations of the analyte are far below. Additionally, intra- and inter-run coefficients of variation are presented under a usual way in Table 1. Finally, bAla, EtN, and PSer show the worst precision index with highest coefficient of variation.
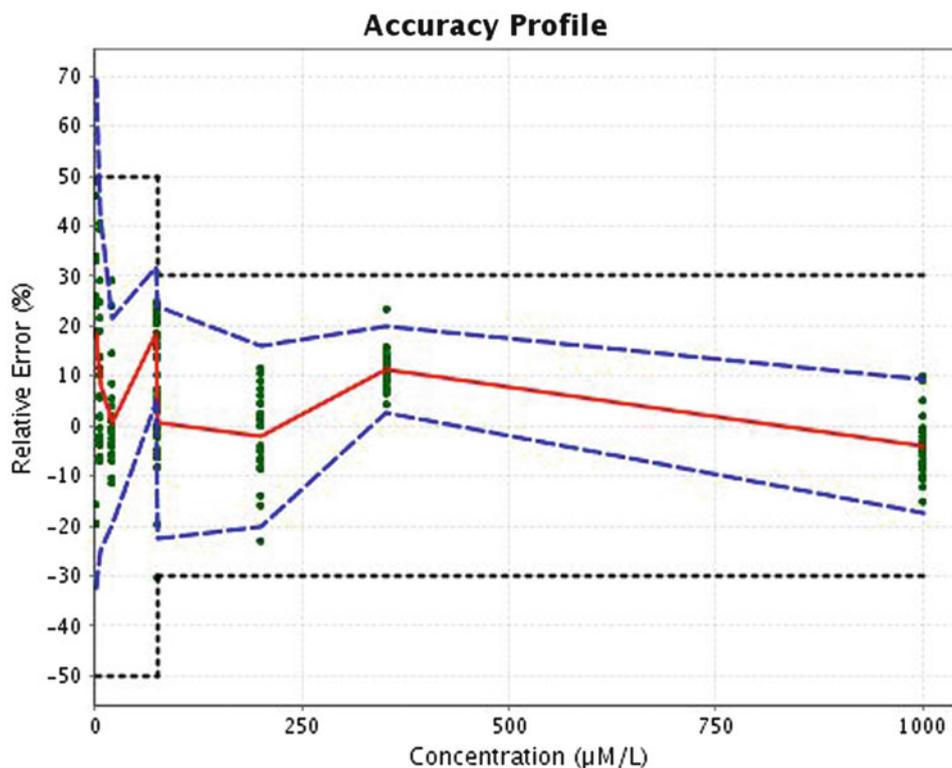
Fig. 1 Accuracy profile of phenylalanine. Plain *red line* is the relative bias, *dashed blue lines* are the β-expectation tolerance limit ($\beta = 5$ %), and dotted black curves represent the acceptance limit (±50 % for the concentration smaller than 75 μmol/L and ±30 % for concentration equal or greater than this value). The *dots* represent the relative back-calculated concentrations of the validation standards and are plotted according to their targeted concentration

MS Versus IEC

Results of method comparison are presented in Table 2. For all amino acids tested, slopes and intercepts are very close to 1 and 0, respectively.

Normal Population Values

Reference intervals have been established on plasma, urine, and CSF samples collected from normal controls (Table 3). For each, ranges were defined to cover the central 90 % of the measured values, and for plasma and urine matrices, three age classes have been considered.

In urine, the concentration of each amino acid was normalized against creatinine and then expressed in mmol/mol of creatinine. As previously described (Illsinger et al. 2010), large variations in amino acid concentrations are observed in urine of newborns, reflecting the variability of renal tubular function in the first months of life (Rossi et al. 1994).

Medical Valuation

Samples, collected from confirmed IEM patients, revealed positive results, according to their respective pathology (Table 4).

Discussion

We present here an exhaustive assessment of the aTRAQ kit for Amino Acid Analysis of Physiological Fluids, using a total error approach. To our knowledge, this is the first validation report of amino acid profiling through the use of accuracy profile.

Based on the most recent manufacturer's recommendations, a few modifications have been appended to the original protocol for sample preparation. These improvements are the consequence of some troubles reported by customers in methionine quantification with the native procedure.

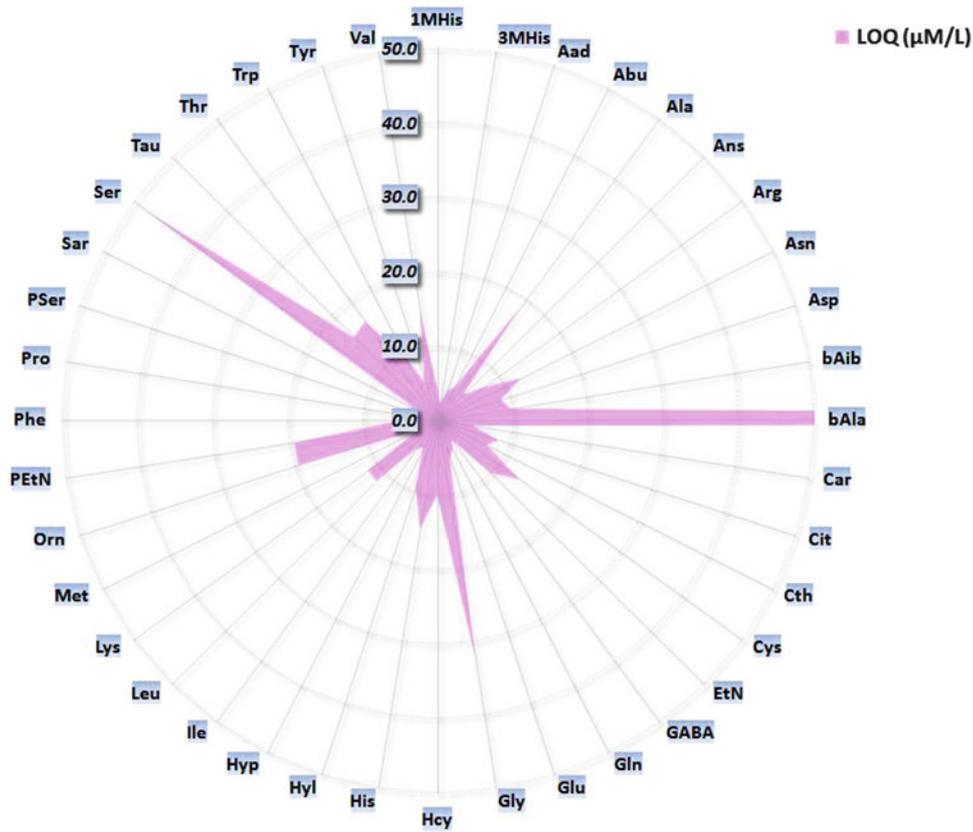**Fig. 2** Radar chart of calculated LLOQ, based on the accuracy profile approach
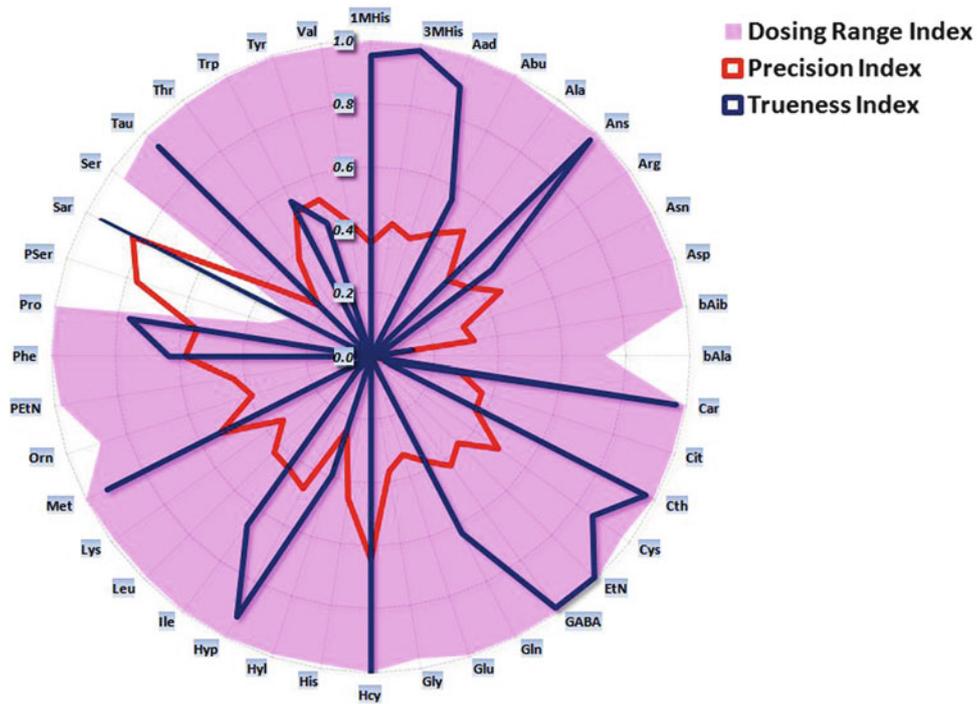

Fig. 3 Radar chart of each amino acid indexes. Indexes vary from 0 to 1; the better is the method for a criteria, the closer to one is the corresponding index. Compared to the other analytes, dosing range for Asp, Glu, Pser, and Sar are reduced, the instrument detector saturating at higher concentrations

Table 1 Intra- and inter-run variation for two levels of concentration

| | Concentration level – 20 μmol/L | | Concentration level – 200 μmol/L | |
| | Cystine – 10 μmol/L | | Cystine – 100 μmol/L | |
	Intra-run coefficient of variation (%)	Inter-run coefficient of variation (%)	Intra-run coefficient of variation (%)	Inter-run coefficient of variation (%)
1MHis	10.55	11.43	7.88	7.99
3MHis	9.11	9.36	8.54	8.54
Aad	10.93	11.17	8.16	8.16
Abu	8.85	11.48	8.41	9.30
Ala	10.66	11.84	9.42	9.42
Ans	10.78	11.47	7.70	8.31
Arg	11.45	13.11	9.05	9.40
Asn	10.01	10.01	7.27	7.43
Asp	18.72	18.72	6.96	6.96
bAib	11.46	12.19	7.84	7.99
bAla	26.84	41.24	8.38	9.02
Car	12.31	12.31	8.70	8.70
Cit	9.78	10.26	9.14	9.64
Cth	14.54	15.55	8.91	9.44
Cys	11.81	12.94	6.71	9.00
EtN	7.99	15.81	16.21	16.21
GABA	9.82	11.32	7.19	7.19
Gln	10.83	10.85	7.42	7.42
Glu	12.49	13.06	7.15	7.68
Gly	9.85	18.13	10.31	10.31
Hcy	5.02	38.29	17.16	17.16
His	9.61	9.61	10.92	10.92
Hyl	14.30	14.30	10.07	10.36
Hyp	12.14	13.05	3.93	4.39
Ile	12.55	12.55	8.67	8.72
Leu	15.02	15.02	7.79	7.85
Lys	10.73	11.99	9.00	9.00
Met	9.49	10.67	8.09	8.09
Orn	14.47	16.49	7.61	7.72
PEtN	11.51	13.92	7.09	7.25
Phe	9.28	9.71	8.57	8.57
Pro	8.84	9.24	5.82	5.82
PSer	28.71	28.71	10.50	10.50
Sar	10.53	11.25	8.25	8.25
Ser	16.16	18.77	9.06	9.06
Tau	7.23	14.42	3.53	8.36
Thr	9.52	10.24	7.15	8.47
Trp	7.87	9.59	7.54	7.78
Tyr	12.04	14.47	10.76	10.76
Val	10.51	10.91	9.27	9.41

Table 2 Method comparison: IEC versus MS method. Slope and intercept were calculated by means of a nonparametric Passing and Bablok regression

	Slope	Slope 95 % CI[a]	Intercept	Intercept 95 % CI[a]
3MHis	1.01	0.938 to 1.362	3.19	−3.863 to 5.464
Abu	1.17	1.105 to 1.252	−2.94	−5.209 to 0.857
Ala	1.05	1.016 to 1.085	−7.32	−20.1 to 2.336
Arg	1.06	1.045 to 1.083	−0.92	−5.514 to 1.503
Asn	1.19	1.151 to 1.279	5.70	−5.066 to 9.417
Asp	0.99	0.927 to 1.071	1.06	−2.051 to 2.432
Cit	0.96	0.938 to 1.008	1.88	0.449 to 3.583
Cth	1.12	1.005 to 1.156	0.55	−0.428 to 1.388
Cys	1.08	1.011 to 1.167	−3.07	−6.464 to 0.94
Gln	0.92	0.803 to 1.083	3.72	−33.91 to 9.132
Glu	1.08	1.041 to 1.132	3.38	−2.056 to 6.867
Gly	0.98	0.928 to 1.058	1.28	−15.09 to 1.45
His	1.01	0.98 to 1.071	−0.12	−5.099 to 2.776
Hpro	1.14	1.097 to 1.223	−0.39	−4.912 to 2.063
Ile	1.09	0.995 to 1.188	1.19	−2.022 to 5.713
Leu	1.11	1.001 to 1.094	1.16	−5.852 to 6.11
Lys	1.02	1.001 to 1.094	−4.87	−11.99 to 0.824
Met	1.08	1.072 to 1.173	0.20	−5.297 to 0.879
Orn	1.03	0.986 to 1.097	−5.23	−12.84 to 0.285
Phe	1.18	1.051 to 1.294	−4.46	−10.86 to 3.971
Pro	1.18	1.141 to 1.226	6.21	0.419 to 9.499
Ser	1.15	1.087 to 1.246	1.06	−5.565 to 4.681
Tau	1.08	1.022 to 1.154	−10.95	−21.82 to 2.961
Thr	1.01	0.965 to 1.105	4.28	−4.631 to 9.206
Trp	1.02	0.928 to 1.124	3.40	−5.842 to 2.067
Tyr	1.00	0.927 to 1.05	0.64	−3.441 to 7.371
Val	1.07	1.035 to 1.117	−3.31	−22.83 to 4.374

[a] 95 % CI = Confidence interval at 95 % for slope and intercept, respectively

1. The first amelioration referred to the addition of the aTRAQ Internal Standard solution before the evaporation step, and not after as required in the initial method. During this dryness stage, an oxidation phenomenon occurs, which mainly affects methionine and cystathionine. As internal standards were initially added after this step, they were not subjected to this degradation, and Met and Cth were then underestimated. Therefore, adding the Internal Standard solution before desiccation equally affects the metabolites to be quantified and their corresponding internal standards.

2. After hydroxylamine pipetting, an additional 15-min incubation step at room temperature was initiated. The phenolic group of tyrosine is also slowly and

Table 3 Amino acid reference ranges on plasma, urine, and CSF matrices

	Plasma			Urine			CSF
	0–2 years μmol/L	*2–12 years* μmol/L	*>= 12 years* μmol/L	*0–2 years* mM/M Creat.	*2–12 years* mM/M Creat.	*>= 12 years* mM/M Creat.	*/* μmol/L
1MHis	0.1–3.7	0.2–15.7	0.5–14	2.7–18.9	2.7–208	3.6–137	0–0.9
3MHis	1.4–5.7	1.3–7.1	2.4–8	7.4–37.5	12.5–35.4	10.2–25.7	0.1–0.4
Aad	0.6–1.8	0.3–1.6	0.4–2	0.9–46.3	1.4–13.3	0.7–7.4	0–0.3
Abu	3.1–16.7	7.6–28	4.4–36.3	0.4–7.4	0.6–4.9	0.4–2.4	1.3–4
Ala	122–426	111–518	153–592	32.1–235	16.8–129	7.4–53.8	19–32.5
Ans	0–0.9	0–1.1	0–0.7	0–8.3	0–41.8	0–19.2	0–12
Arg	14.4–164	18.4–102	11.2–128	0.7–43.3	6.1–61.7	1.1–11.6	10.8–27
Asa[a]	0.3–7.8	0–13.7	0–12.5	0–18.2	1.1–4.2	0.5–3	0.4–6.7
Asn	27.1–123	28.4–102	22.7–115	3.3–136	8.4–48	3.5–36.6	6.6–12.1
Asp	6.1–69.4	4.6–60.3	4.4–65	0.8–20.3	0.3–9.4	0.3–4.6	1–11.6
bAib	0.8–31.8	0.5–3.3	0.3–2.9	3–98.1	2.6–107.2	1.5–27.6	0–0.2
bAla	17.2–67.3	15.8–49.9	18.5–60.7	1.5–116	0.3–15.9	0.2–13.7	17–41.4
Car	0–2.2	0–0.4	0–0.5	1.3–62	2–43.1	0.2–12.1	0–0.1
Cit	7.9–33.6	8.4–40.7	13.5–63.3	0.3–18.5	0.3–2.1	0.1–1.7	1.9–20
Cth	0.3–29.9	0.1–1.2	0.1–1.5	0.7–13	0.3–5	0.3–4.4	0.1–0.5
Cys	6.4–124	0.9–39.1	7.3–58.5	2.5–91.7	2.1–9.8	1.7–10	0–1
EtN	3.8–46.6	4.1–21.9	3.8–24.6	12.3–149	21.7–69.3	15.1–34.8	4.7–41.3
GABA	0.2–57.7	0.2–4.7	0.2–6.5	0.2–5.3	0.2–1.3	0.1–0.7	0.2–2.1
Gln	336–691	300–688	296–884	3–357	32–134	15–90	374–836
Glu	38–353	35–288	41.9–236	2.5–42.6	1.3–37.5	0.9–10.7	0.9–12
Gly	113–458	164.7–402	94.9–463	79.2–1,057	66–417	43–360	9.3–34
Hcit[a]	0.1–0.7	0–0.8	0.1–0.7	0.3–29.4	1–8.1	0.8–3.9	0.1–0.4
Hcy	0–26.3	0–0.4	0–0.3	0–1.8	0–0.2	0–0.2	0–0.5
His	35–117	34.2–114	35.4–136.2	46–379	34.8–290	18–125	9.2–25.3
Hyl	0.1–110	0.1–86.4	0–103.2	1.1–40.3	0.5–11	0.1–1.7	0.1–2
Hyp	10.8–76.2	8.7–31.9	4.8–34.6	1.4–233	0.4–4.2	0.1–2.3	0.3–1.8
Ile	30.3–129	24.7–94.7	28.3–167.8	1–12	1–4.2	0.2–2.6	3.2–12.9
Leu	44.9–198	44.5–158	61.2–203.8	0.6–21.1	2.1–9.9	0.3–6.6	5–10
Lys	64.5–375	75.5–228	105–253	2–274	4.7–105	1.9–23.5	6.3–28.6
Met	9.4–67.6	9.3–31	9.6–44.4	0.2–5.1	0.5–2.3	0–1.3	1.5–4.2
Orn	19.9–181	23.4–165	42.8–186	1.5–52.8	0.9–5.4	0.6–2.6	4.5–10
PEtN	0–6	0–3.8	0.2–2.3	1.8–28.5	2.5–22.1	0.6–4.9	0.8–5.6
Phe	25.5–131	27.7–95.8	23.5–104	1.4–38.2	4.2–19	1.9–10.1	6.3–13.5
Pro	74.9–276	89.2–286	85.8–327.8	1.9–97.2	0.6–9.5	0.3–5	1.4–5.3
PSer	0–0.4	0–0.4	0–0.4	0–1.2	0–0.3	0–0.2	0–0.3
Sar	0.4–3	0.8–4.7	0.5–2.7	0.2–12.4	0–1.2	0–1.3	0–0.2
Ser	62–253	61.8–230	53.7–216	5.3–302	26.4–90.6	11.1–52.6	25.3–67.1
Tau	34.8–309	34.9–266	30.3–223	6–546	14.6–349	4.5–140	4.5–10.1
Thr	41.7–252	51–167	58.3–206	4.8–171	7.5–40.5	4.5–35	15.3–36.1
Trp	17.1–87.3	15.1–58.4	24.6–85.7	2.2–33.7	4–21.1	2.4–11	1.3–3.2
Tyr	24–154	27.3–92.1	34–101	1.3–76.2	6.2–45.5	2.8–17.9	6.9–17.8
Val	72.6–277	94.8–261	99.2–329	1.2–24.7	3.1–12	1–7.9	8.3–21.5

[a] Asa and Hcit concentrations were calculated based upon the corresponding IS concentrations, as they were not included in the five-point external calibration curve

Table 4 Amino acid concentrations measured in different IEM

IEM	Matrix	Amino acid	Concentration (age-related normal range)
MSUD	Plasma	Leu	491 μmol/L (61,0–341,0)
		allo-Ile[a]	115 μmol/L
PKU	Plasma	Phe	1,230 μmol/L (23,5–104,0)
Ornithinemia	Plasma	Orn	660 μmol/L (42.8–346.2)
Treated tyrosinemia type I	Urine	Tyr	86,8 mM/M creatinine (2,8–17,9)
NKHG[b]	Urine	Gly	3971 mM/M creatinine (66,0–416,7)
Cystinuria	Urine	Arg	157,6 mM/M creatinine (6,1–61,7)
		Cys	93,1 mM/M creatinine (2,1–9,8)
		Lys	551,0 mM/M creatinine (4,7–105,0)
		Orn	148,8 mM/M creatinine (0,9–5,4)
Cystathioninuria	Urine	Cth	208 mM/M creatinine (0,3–4,4)

[a] allo-Isoleucine

[b] Non ketotic hyperglycinemia

partially labeled by the aTRAQ Δ8-reagent. Hydroxylamine solution is used to remove this extra label, but as internal standard is added before the drying step, the concentration of hydroxylamine is reduced compared to the original protocol. This dilution slows down the reaction removing the unwanted tag and, therefore, an extra incubation time is required.

3. Finally, the evaporation step under nitrogen stream was reduced to the half mixture volume, and not to dryness as requested by the original proceeding. Indeed, extended drying can result in excessive methionine oxidation.

This latter protocol gives satisfaction for amino acids profiling. Sulfur-containing amino acids generate adequate results with reliable recoveries for Met and Cth. β-Alanine is poorly reproducible on lower concentrations. Because of detector saturation at higher concentrations, the limit of quantification of sarcosine was fixed at 272 μmol/L. Finally, phosphoserine gave precise but inaccurate results at higher concentrations (>341 μmol). Reference medical ranges are equivalent with published intervals (Duran 2008). Results comparison with IEC showed a right concordance, and patients affected by IEM have been readily identified.

Conclusion

The presented method is focused on the targeted identification of about 40 amino acids. The application has been evaluated and offers a valid alternative in a clinical context. Furthermore, the present approach offers certain advantage compared to the IEC method: lower sample volume, time run reduction, and better specificity. Additionally, the use of labeled internal standards for each amino acid confers reliable quantitative results. Neglecting maintenance costs of a devoted IEC-instrument, analytical running expenses are similar between the two methods.

Nevertheless, not everything is idyllic. Although chromatography time drops down from 180 to 18 min, the aTRAQ method requires much more of that time difference for extraction, derivatization, and data processing, this latter step being labor intensive. In addition, some specific ninhydrin-reactive species (i.e., aspartylglucosamine or pipecolic acid), which may be identified with any IEC amino acid analyzer, will remain undetectable with the MRM approach. Nonetheless, implementation of additional analytes in the MS acquisition method should be a future concern. Besides, this latter consideration depicts a certain advantage of the MS technology, as these "extra-analytes" should not involve exclusively ninhydrin-responsive compounds.

Aware that the sensitivity of ultimate generation mass spectrometer is far below major physiological amino acid concentrations, we demonstrate here that these technological advances, which are fundamental for traces detection (on the order of picomol/L or lower), are not incompatible with the reliable quantification of elevated amino acid concentrations (i.e., >100 μmol/L). Therefore, running methods requiring very high sensitivity can be easily combined with less sensitive protocols on the same instruments.

Finally, the aTRAQ kit is a very good alternative to the IEC method, but financial and practical considerations of both technologies have to be counterbalanced before engaging any transition.

Synopsis

Validation of the aTRAQ assay for Amino Acid Analysis in Physiological Fluids.

Compliance with Ethics Guidelines

Disclosure

Romain FILEE, Roland SCHOOS, and François BOEMER declare no conflict of interest.

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